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(54) Title: NUCLEOTIDE SEQUENCES FOR THE TAL GENE

(57) Abstract: The invention relates to an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence chosen from the group consisting of a) polynucleotide which is identical to the extent of at least 70 % to a polynucleotide which codes for a polypeptide which comprises the amino acid sequences of SEQ ID NO. 2 or SEQ ID NO. 4, b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70 % to the amino acid sequences of SEQ ID NO. 2 or SEQ ID NO. 4, c) polynucleotide which is complementary to the polynucleotides of a) or b) and, d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequences of a), b) or c) and a process for the preparation of L-amino acids, which comprises carrying out the following steps: a) fermentation of the desired L-amino acid-producing bacteria in which at least the tal gene is amplified, b) concentration of the desired product in the medium or in the cells of the bacteria and c) isolation of the L-amino acid.

NUCLEOTIDE SEQUENCES FOR THE TAL GENE

The invention provides nucleotide sequences which code for the tal gene and a process for the fermentative preparation of amino acids, in particular L-lysine, L-threonine, L-
5 isoleucine and L-tryptophan, using coryneform bacteria in which the tal gene is amplified.

Prior art --

Amino acids, in particular L-lysine, are used in human
medicine and in the pharmaceuticals industry, but in
10 particular in animal nutrition.

It is known that amino acids are prepared by fermentation by strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great
importance, work is constantly being undertaken to improve
15 the preparation processes. Improvements to the processes can relate to fermentation measures, such as e. g. stirring and supply of oxygen, or the composition of the nutrient media, such as e. g. the sugar concentration during the fermentation, or the working up to the product form by
20 e. g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to
25 antimetabolites, such as e. g. the lysine analogue S-(2-aminoethyl)-cysteine, or are auxotrophic for metabolites of regulatory importance and produce L-amino acids, such as e. g. L-lysine, are obtained in this manner.

Methods of the recombinant DNA technique have also been
30 employed for some years for improving the strain of Corynebacterium strains which produce amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

Review articles in this context are to be found, inter alia, in Kinoshita ("Glutamic Acid Bacteria", in: Biology of Industrial Microorganisms, Demain and Solomon (Eds.), Benjamin Cummings, London, UK, 1985, 115-142), Hilliger 5 (BioTec 2, 40-44 (1991)), Eggeling (Amino Acids 6:261-272 (1994)), Jetten and Sinskey (Critical Reviews in Biotechnology 15, 73-103 (1995)) and Sahm et al. (Annals of the New York Academy of Science 782, 25-39 (1996)).

The importance of the pentose phosphate cycle for the 10 biosynthesis and production of amino acids, in particular L-lysine, by coryneform bacteria is the subject of numerous efforts among experts.

Thus Oishi and Aida (Agricultural and Biological Chemistry 29, 83-89 (1965)) report on the "hexose monophosphate 15 shunt" of *Brevibacterium ammoniagenes*. Sugimoto and Shio (Agricultural and Biological Chemistry 51, 101-108 (1987)) report on the regulation of glucose 6-phosphate dehydrogenase in *Brevibacterium flavum*.

Object of the invention

20 The inventors had the object of providing new measures for improved fermentative preparation of amino acids, in particular L-lysine, L-threonine, L-isoleucine and L-tryptophan.

Description of the invention

25 Amino acids, in particular L-lysine, are used in human medicine, in the pharmaceuticals industry and in particular in animal nutrition. There is therefore a general interest in providing new improved processes for the preparation of amino acids, in particular L-lysine.

30 When L-lysine or lysine are mentioned in the following, not only the base but also the salts, such as e. g. lysine

monohydrochloride or lysine sulfate, are also meant by this.

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence
5 chosen from the group consisting of

- a) polynucleotide which is identical to the extent of at least 70 % to a polynucleotide which codes for a polypeptide which comprises the amino acid sequences of SEQ ID NO. 2 or SEQ ID NO. 4,
- 10 b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequences of SEQ ID NO. 2 or SEQ ID NO. 4,
- c) polynucleotide which is complementary to the
15 polynucleotides of a) or b) and
- d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c).

The invention also provides the polynucleotide as claimed
20 in claim 1, this preferably being a DNA which is capable of replication, comprising:

- (i) a nucleotide sequence chosen from the group consisting of SEQ ID NO. 1 and SEQ ID NO. 3 or
- (ii) at least one sequence which corresponds to
25 sequence (i) within the range of the degeneration of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally
- 30 (iv) sense mutations of neutral function in (i).

The invention also provides

a polynucleotide as claimed in claim 4, comprising one of the nucleotide sequences as shown in SEQ ID NO. 1 and SEQ ID NO. 3,

5 a polynucleotide as claimed in claim 5, which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID NO. 2 and SEQ ID NO. 4,

a vector containing the polynucleotide as claimed in claim 1,

10 and coryneform bacteria, serving as the host cell, which contain the vector.

The invention also provides polynucleotides which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a
15 corresponding gene library, which comprises the complete gene with the polynucleotide sequence corresponding to SEQ ID NO. 1 or SEQ ID NO. 3, with a probe which comprises the sequence of the polynucleotide mentioned, according to

SEQ ID NO. 1 or SEQ ID NO. 3 or a fragment thereof, and
20 isolation of the DNA sequence mentioned.

Polynucleotide sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, cDNA which code for transaldolase and to isolate those cDNA or genes which have
25 a high similarity of sequence with that of the transaldolase gene.

Polynucleotide sequences according to the invention are furthermore suitable as primers for the preparation of DNA of genes which code for transaldolase by the polymerase
30 chain reaction (PCR).

Such oligonucleotides which serve as probes or primers comprise at least 30, preferably at least 20, especially preferably at least 15 successive nucleotides.

Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

10 "Polypeptides" is understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID NO. 2 or SEQ ID NO. 4, in particular those with the biological activity of transaldolase, and also those which are identical to the extent of at least 70 % to the polypeptide according to SEQ ID NO. 2 or SEQ ID NO. 4, and preferably are identical to the extent of at least 80% and in particular to the extent of at least 90 % to 95 % to the polypeptide according to SEQ ID NO. 2 or SEQ ID NO. 4, and have the activity mentioned.

The invention also provides a process for the fermentative preparation of amino acids, in particular L-lysine, L-threonine, L-isoleucine and L-tryptophan, using coryneform bacteria which in particular already produce an amino acid, and in which the nucleotide sequences which code for the tal gene are amplified, in particular over-expressed.

The term "amplification" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or

using a gene which codes for a corresponding enzyme having a high activity, and optionally combining these measures.

The microorganisms which the present invention provides can prepare L-amino acids, in particular L-lysine, from
5 glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species
10 *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum*, are, for example, the known wild-type strains

- 15 *Corynebacterium glutamicum* ATCC13032
- Corynebacterium acetoglutamicum* ATCC15806
- Corynebacterium acetoacidophilum* ATCC13870
- Corynebacterium thermoaminogenes* FERM BP-1539
- Corynebacterium melassecola* ATCC17965
- 20 *Brevibacterium flavum* ATCC14067
- Brevibacterium lactofermentum* ATCC13869 and
- Brevibacterium divaricatum* ATCC14020

and L-lysine-producing mutants or strains prepared therefrom, such as, for example

- 25 *Corynebacterium glutamicum* FERM-P 1709
- Brevibacterium flavum* FERM-P 1708
- Brevibacterium lactofermentum* FERM-P 1712
- Corynebacterium glutamicum* FERM-P 6463
- Corynebacterium glutamicum* FERM-P 6464 and
- 30 *Corynebacterium glutamicum* ATCC13032
- Corynebacterium glutamicum* DM58-1
- Corynebacterium glutamicum* DSM12866.

and L-threonine-producing mutants or strains prepared therefrom, such as, for example

- Corynebacterium glutamicum ATCC21649
- Brevibacterium flavum BB69
- 5 Brevibacterium flavum DSM5399
- Brevibacterium lactofermentum FERM-BP 269
- Brevibacterium lactofermentum TBB-10

and L-isoleucine-producing mutants or strains prepared therefrom, such as, for example

- 10 Corynebacterium glutamicum ATCC 14309
- Corynebacterium glutamicum ATCC 14310
- Corynebacterium glutamicum ATCC 14311
- Corynebacterium glutamicum ATCC 15168
- Corynebacterium ammoniagenes ATCC 6871

- 15 and L-tryptophan-producing mutants or strains prepared therefrom, such as, for example

- Corynebacterium glutamicum ATCC21850 and
 - Corynebacterium glutamicum KY9218 (pKW9901)

- The inventors have succeeded in isolating the new tal gene
- 20 of *C. glutamicum* which codes for transaldolase (EC 2.2.1.2).

- To isolate the tal gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in *E. coli*. The setting up of gene libraries is
- 25 described in generally known textbooks and handbooks. The textbook by Winnacker: *Gene und Klone, Eine Einführung in die Gentechnologie* [Genes and Clones, An Introduction to Genetic Engineering] (Verlag Chemie, Weinheim, Germany, 1990) or the handbook by Sambrook et al.: *Molecular*
- 30 *Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the *E. coli* K-12 strain W3110 set

up in λ vectors by Kohara et al. (Cell 50, 495-508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575). Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992) in turn describe a gene library of *C. glutamicum* ATCC13032 using the cosmid pH79 (Hohn and Collins, Gene 11, 291-298 (1980)). O'Donohue (The Cloning and Molecular Analysis of Four Common Aromatic Amino Acid Biosynthetic Genes from *Corynebacterium glutamicum*. Ph.D. Thesis, National University of Ireland, Galway, 1997) describes the cloning of *C. glutamicum* genes using the λ Zap expression system described by Short et al. (Nucleic Acids Research, 16: 7583). To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are, in particular, those *E. coli* strains which are restriction- and recombination-defective. An example of these is the strain DH5 α mc^r, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can then in turn be subcloned and subsequently sequenced in the usual vectors which are suitable for sequencing, such as is described e. g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The DNA sequences obtained can then be investigated with known algorithms or sequence analysis programs, such as e. g. that of Staden (Nucleic Acids Research 14, 217-232(1986)), the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)) the FASTA algorithm

of Pearson and Lipman (Proceedings of the National Academy of Sciences USA 85,2444-2448 (1988)) or the BLAST algorithm of Altschul et al. (Nature Genetics 6, 119-129 (1994)) and compared with the sequence entries which exist in databanks
5 accessible to the public. Databanks for nucleotide sequences which are accessible to the public are, for example, that of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany) of that of the National Center for Biotechnology Information (NCBI,
10 Bethesda, MD, USA).

The invention provides the new DNA sequence from C.glutamicum which contains the DNA section which codes for the tal gene, shown as SEQ ID NO 1 and SEQ ID NO 3. The amino acid sequence of the corresponding protein has
15 furthermore been derived from the present DNA sequence using the methods described above. The resulting amino acid sequence of the tal gene product is shown in SEQ ID NO 2 and SEQ ID NO 4.

A gene library produced in the manner described above can
20 furthermore be investigated by hybridization with nucleotide probes of known sequence, such as, for example, the zwf gene (JP-A-09224661). The cloned DNA of the clones which show a positive reaction in the hybridization is sequenced in turn to give on the one hand the known
25 nucleotide sequence of the probe employed and on the other hand the adjacent new DNA sequences.

Coding DNA sequences which result from SEQ ID NO 3 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which
30 hybridize with SEQ ID NO 3 or parts of or SEQ ID NO 3 are a constituent of the invention. Conservative amino acid exchanges, such as e. g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which
35 do not lead to a fundamental change in the activity of the

protein, i.e. are of neutral function. It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found
5 by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and
10 molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID NO 2 or SEQ ID NO 4 are also a constituent of the invention.

In the same way, DNA sequences which hybridize with or SEQ ID NO 3 or parts of or SEQ ID NO 3 are a constituent of the
15 invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID NO 3 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

20 Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal
25 of Systematic Bacteriology (1991) 41: 255-260). Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonukleotide synthesis: a practical approach (IRL Press,
30 Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

The inventors have found that coryneform bacteria produce amino acids in an improved manner after over-expression of the tal gene.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes
5 which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-amino acid production. The expression is likewise improved by measures to prolong the life of the m-
10 RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-
15 expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146
20 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European Patent Specification EPS 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in
25 Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15 - 24 (1993)), in Japanese Laid-Open Specification JP-A-10-
30 229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

By way of example, the tal gene according to the invention
35 was over-expressed with the aid of plasmids.

Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as e. g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., 5 Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as e. g. those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 10 (1990)), or pAG1 (US-A 5,158,891), can be used in the same manner.

Plasmid vectors which are furthermore suitable are also those with the aid of which the process of gene amplification by integration into the chromosome can be 15 used, as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for duplication or amplification of the hom-thrB operon. In this method, the complete gene is cloned in a plasmid vector which can replicate in a host (typically E. 20 coli), but not in C. glutamicum. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological 25 Chemistry 269:32678-84; US-A 5,487,993), pCR@Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342). The plasmid vector 30 which contains the gene to be amplified is then transferred into the desired strain of C. glutamicum by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for 35 transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362

(1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene in question.

An example of a plasmid vector with the aid of which the process of amplification by integration can be carried out is pSUZ1, which is shown in Figure 1. Plasmid pSUZ1 consists of the E. coli vector pBGS8 described by Spratt et al. (Gene 41: 337-342(1986)), into which the tal gene has been incorporated.

In addition, it may be advantageous for the production of amino acids to amplify or over-express one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the pentose phosphate pathway or of amino acid export, in addition to the tal gene.

Thus, for example, for the preparation of L-amino acids, in particular L-lysine, one or more genes chosen from the group consisting of

- 20 • the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- the lysC gene which codes for a feed back resistant aspartate kinase (Kalinowski et al. (1990), Molecular and General Genetics 224: 317-324),
- 25 • the gap gene which codes for glycerolaldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
- 30 • the mqo gene which codes for malate:quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),

- the tkt gene which codes for transketolase (accession number AB023377 of the databank of European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany)),
- the gnd gene which codes for 6-phosphogluconate
5 dehydrogenase (JP-A-9-224662),
- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-9-224661),
- the lysE gene which codes for lysine export (DE-A-195 48 222),
- 10 • the zwf gene (DE 199 59 328.0; DSM 13115),
- the eno gene which codes for enolase (DE: 19947791.4),
- the devB gene,
- the opcA gene (DSM 13264)

can be amplified, preferably over-expressed, at the same
15 time.

Thus, for example, for the preparation of L-threonine, one or more genes chosen from the group consisting of

- at the same time the hom gene which codes for homoserine dehydrogenase (Peoples et al., Molecular Microbiology 2,
20 63-72 (1988)) or the hom^{dr} allele which codes for a "feed back resistant" homoserine dehydrogenase (Archer et al., Gene 107, 53-59 (1991),
- the gap gene which codes for glycerolaldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology
25 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),

- the mgo gene which codes for malate:quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the tkt gene which codes for transketolase (accession
5 number AB023377 of the databank of European Molecular
Biologies Laboratories (EMBL, Heidelberg, Germany)),
- the gnd gene which codes for 6-phosphogluconate dehydrogenase (JP-A-9-224662),
- the zwf gene which codes for glucose 6-phosphate
10 dehydrogenase (JP-A-9-224661),
- the thrE gene which codes for threonine export (DE 199 41
478.5; DSM 12840),
- the zwf1 gene (DE 199 59 328.0; DSM 13115),
- the eno gene which codes for enolase (DE: 19947791.4),
- 15 • the devB gene,
- the opcA gene (DSM 13264)

can be amplified, preferably over-expressed, at the same time.

It may furthermore be advantageous for the production of
20 amino acids to attenuate

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1 DSM 13047) and/or
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969), or
- 25 • the poxB gene which codes for pyruvate oxidase (DE 199 51 975.7; DSM 13114), or

- the *zwa2* gene (DE: 199 59 327.2; DSM 13113)

at the same time, in addition to the amplification of the *tal* gene.

In addition to over-expression of the *tal* gene it may
5 furthermore be advantageous for the production of amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

- 10 The microorganisms prepared according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of L-amino acids. A summary of
15 known culture methods are described in the textbook by Chmiel (Bioprozeßtechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und
20 periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

- The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained
25 in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981). Sugars and carbohydrates, such as e. g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as
30 e. g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e. g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e. g. glycerol and ethanol, and organic acids, such as e. g. acetic acid, can be used as the source of carbon. These substances can be

used individually or as a mixture. Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulphate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture. Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.

Antifoams, such as e. g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e. g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e. g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of L-amino acid has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190).

- 5 The following microorganism has been deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:
- 10 • *Escherichia coli* JM109/pSUZ1 as DSM 13263.

SEQ ID NO 1 also contains the new devB gene. The process according to the invention is used for fermentative preparation of amino acids.

The following figures are attached:

Figure 1: Map of the plasmid pSUZ1

The abbreviations and designations used have the following meaning.

- | | | |
|----|----------|---|
| 5 | lacZ: | segments of lacZ α gene fragment |
| | kan r: | kanamycin resistance |
| | tal: | transaldolase gene |
| | ori: | origin of replication of plasmid pBGS8 |
| | BclI: | cleavage site of restriction enzyme BclI |
| 10 | EcoRI: | cleavage site of restriction enzyme EcoRI |
| | HindIII: | cleavage site of restriction enzyme HindIII |
| | PstI: | cleavage site of restriction enzyme PstI |
| | SacI: | cleavage site of restriction enzyme SacI |

Examples

The following examples will further illustrate this invention. The molecular biology techniques, e.g. plasmid DNA isolation, restriction enzyme treatment, ligations, 5 standard transformations of *Escherichia coli* etc. used are, (unless stated otherwise), described by Sambrook et al., (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbour Laboratories, USA).

Example 1

- 10 Preparation of a genomic cosmid gene library from
Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from *Corynebacterium glutamicum* ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme 15 Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid 20 vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, 25 Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase. The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). 30 The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of

Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217). For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575)

5 the cells were taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on

10 LB agar (Lennox, 1955, Virology, 1:190) with 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 2

Isolation and sequencing of the tal gene

15 The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product

20 Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid

25 fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany). The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Holland, Product Description Zero Background Cloning Kit,

30 Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al.

35 (1989, Molecular Cloning: A laboratory Manual, Cold Spring

Harbor), the DNA mixture being incubated overnight with T4
ligase (Pharmacia Biotech, Freiburg, Germany). This
ligation mixture was then electroporated (Tauch et al.
1994, FEMS Microbiol Letters, 123:343-7) into the E. coli
5 strain DH5 α MCR (Grant, 1990, Proceedings of the National
Academy of Sciences U.S.A., 87:4645-4649) and plated out on
LB agar (Lennox, 1955, Virology, 1:190) with 50 μ g/ml
zeocin. The plasmid preparation of the recombinant clones
was carried out with Biorobot 9600 (Product No. 900200,
10 Qiagen, Hilden, Germany). The sequencing was carried out by
the dideoxy chain-stopping method of Sanger et al. (1977,
Proceedings of the National Academy of Sciences U.S.A.,
74:5463-5467) with modifications according to Zimmermann et
al. (1990, Nucleic Acids Research, 18:1067). The "RR
15 dRhodamin Terminator Cycle Sequencing Kit" from PE Applied
Biosystems (Product No. 403044, Weiterstadt, Germany) was
used. The separation by gel electrophoresis and analysis of
the sequencing reaction were carried out in a "Rotiphoresis
NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No.
20 A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377"
sequencer from PE Applied Biosystems (Weiterstadt,
Germany).

The raw sequence data obtained were then processed using
the Staden program package (1986, Nucleic Acids Research,
25 14:217-231) version 97-0. The individual sequences of the
pZero1 derivatives were assembled to a continuous contig.
The computer-assisted coding region analysis were prepared
with the XNIP program (Staden, 1986, Nucleic Acids
Research, 14:217-231). Further analyses were carried out
30 with the "BLAST search program" (Altschul et al., 1997,
Nucleic Acids Research, 25:3389-3402), against the non-
redundant databank of the "National Center for
Biotechnology Information" (NCBI, Bethesda, MD, USA).

The nucleotide sequence obtained is shown in SEQ ID NO 1
35 and SEQ ID NO 3.

Example 3

Cloning of the tal gene

PCR was used to amplify DNA fragments containing the entire tal gene of *C. glutamicum* 13032 and flanking upstream and downstream regions. PCR reactions were carried out using oligonucleotide primers designed from the sequence as determined in Examples 1 and 2. Genomic DNA was isolated from *Corynebacterium glutamicum* ATCC13032 according to Heery and Dunican (Applied and Environmental Microbiology 59: 791-799 (1993)) and used as template. The tal primers used were:

fwd. primer: 5' GGT ACA AAG GGT CTT AAG 3'C

rev. primer: 5' GAT TTC ATG TCG CCG TTA 3'

PCR Parameters were as follows:

15 35 cycles
 95°C for 3 minutes
 94°C for 1 minute
 47°C for 1 minute
 72°C for 45 seconds
20 2.0 mM MgCl₂
 approximately 150-200 ng DNA template.

The PCR product obtained was cloned into the commercially available pGEM-T vector purchased from Promega Corp. (pGEM-T Easy Vector System 1, cat. no. A1360, Promega UK, Southampton, UK) using strain *E. coli* JM109 (Yanisch-Perron et al., Gene, 33: 103-119 (1985)) as a host. The entire tal gene was subsequently isolated from the pGEM T-vector on an Eco RI fragment and cloned into the lacZ α EcoRI site of the *E. coli* vector pBGS8 (Spratt et al., Gene 41(2-3): 337-342 (1986)). The restriction enzymes used were obtained from Boehringer Mannheim UK Ltd. (Bell Lane, Lewes East Sussex BN7 1LG, UK) and used according to manufacturer's instructions. *E. coli* JMI09 was then transformed with this ligation mixture and electrotransformants were selected on

Luria agar supplemented with isopropyl-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-galactopyranoside (XGAL) and kanamycin at concentrations of 1mM, 0.02% and 50 mg/l respectively. Plates were incubated
5 for twelve hours at 37°C. Plasmid DNA was isolated from one transformant, characterised by restriction enzyme analysis using Eco RI. This new construct was designated pSUZ 1.

Example 4

Preparation of the strain *Corynebacterium glutamicum*

10 DSM5715::pSUZ1

The strain DSM5715 was transformed with the plasmid pSUZ1 using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)). Selection of the transformants took place on LBHIS agar comprising
15 18.5 g/l brain-heart infusion broth, 0.5M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

Since the vector pSUZ1 cannot replicate in the strain
20 DSM5715, only clones which show kanamycin resistance imparted by integration of pSUZ1 were able to grow.

The resulting integrant was called DSM5715::pSUZ1.

Example 5

Preparation of lysine

25 The *C. glutamicum* strain DSM5715/pSUZ1 obtained in Example 4 was cultured in a nutrient medium suitable for the production of L-lysine and the L-lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate
30 with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l)) for 24 hours at 33°C. Starting from

this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III:

NaCl	2.5 g/l
Bacto-Peptide	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4.

Kanamycin (25 mg/l) was added to this. The preculture was
5 incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM:

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50g/l
 (NH ₄) ₂ SO ₄	 25 g/l
KH ₂ PO ₄	0.1 g/l
MgSO ₄ * 7 H ₂ O	1.0 g/l
CaCl ₂ * 2 H ₂ O	10 mg/l
FeSO ₄ * 7 H ₂ O	10 mg/l

MnSO ₄ * H ₂ O	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine	0.1 g/l
CaCO ₃	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO₃ 5 autoclaved in the dry state.

Culturing was carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

- 10 After 24 and 48 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange
15 chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	Time, hours	Lysine-HCl g/l
DSM5715	24	8.1
DSM5715::pSUZ1	24	8.6
DSM5715	48	14.7
DSM5715::pSUZ1	48	15.4

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1-1	page	18
1-2	line	5-10
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	26 January 2000 (26.01.2000)
1-3-4	Accession Number	DSMZ 13263
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1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

FOR RECEIVING OFFICE USE ONLY

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FOR INTERNATIONAL BUREAU USE ONLY

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Patent claims

1. An isolated polynucleotide from coryneform bacteria,
comprising a polynucleotide sequence chosen from the
group consisting of
 - 5 a) polynucleotide which is identical to the extent of
at least 70 % to a polynucleotide which codes for a
polypeptide which comprises the amino acid
sequences of SEQ ID NO. 2 or SEQ ID NO. 4,
 - 10 b) polynucleotide which codes for a polypeptide which
comprises an amino acid sequence which is identical
to the extent of at least 70 % to the amino acid
sequences of SEQ ID NO. 2 or SEQ ID NO. 4
 - c) polynucleotide which is complementary to the
polynucleotides of a) or b) and
 - 15 d) polynucleotide comprising at least 15 successive
nucleotides of the polynucleotide sequences of a),
b) or c).
2. A polynucleotide as claimed in claim 1
wherein the polynucleotide is a preferably recombinant
20 DNA which is capable of replication in coryneform
bacteria and additionally contains at least one of the
nucleotide sequences which codes for the genes tkt,
zwf, opcA and devB.
3. A polynucleotide as claimed in claim 1,
25 wherein the polynucleotide is an RNA.
4. A polynucleotide as claimed in claim 2,
comprising one of the nucleotide sequence as shown in
SEQ ID NO. 3.
5. A polynucleotide as claimed in claim 2,
30 which codes for a polypeptide which comprises the

amino acid sequence as shown in SEQ ID NO. 2 and SEQ ID NO. 4.

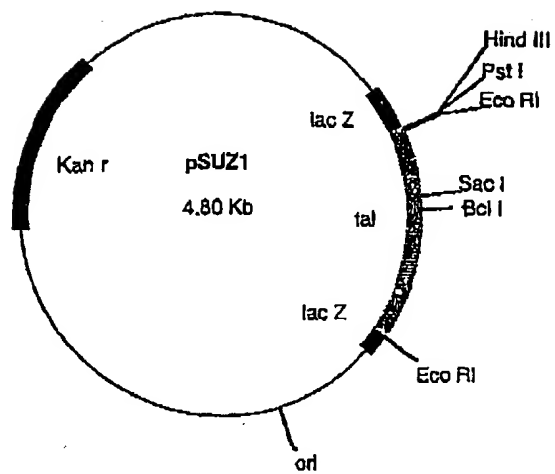
6. A DNA as claimed in claim 2 which is capable of replication, comprising
 - 5 (i) a nucleotide sequence as shown in SEQ ID NO. 3, or
 - (ii) at least one sequence which corresponds to sequences (i) within the range of the degeneration of the genetic code, or
 - 10 (iii) at least one sequence which hybridizes with the sequences complementary to sequences (i) or (ii), and optionally
 - (iv) sense mutations of neutral function in (i).
7. A coryneform bacterium serving as the host cell, which
15 contains a vector which carries a polynucleotide as claimed in claim 1.
8. A process for the preparation of L-amino acids, which comprises carrying out the following steps:
 - 20 a) fermentation of the bacteria which produce the desired L-amino acid, in which at least the tal gene and optionally one or more of the genes tkt gene, zwt gene, devB gene or opcA gene are amplified at the same time,
 - 25 b) concentration of the desired product in the medium or in the cells of the bacteria and
 - c) isolation of the desired L-amino acid.
9. A process as claimed in claim 8, wherein
30 bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally amplified are employed.

10. A process as claimed in claim 8,
wherein
bacteria in which the metabolic pathways which reduce
the formation of the desired L-amino acid are at least
5 partly eliminated are employed.
11. A process as claimed in one or more of claims 8 to 12,
wherein
coryneform bacteria which produce one of the amino
acids from the group consisting of L-lysine, L-
10 threonine, L-isoleucine or L-tryptophan are used.
12. A process for the fermentative preparation of L-amino
acids, in particular lysine, as claimed in claim 8,
wherein
in the coryneform microorganisms which in particular
15 already produce L-amino acids, one or more genes
chosen from the group consisting of
- 12.1 the dapA gene which codes for
dihydrodipicolinate synthase,
- 12.2 the lysC gene which codes for a feed back
20 resistant aspartate kinase,
- 12.3 the gap gene which codes for glycerolaldehyde 3-
phosphate dehydrogenase,
- 12.4 the pyc gene which codes for pyruvate
carboxylase,
- 25 12.5 the mqo gene which codes for malate-quinone
oxidoreductase,
- 12.6 the tkt gene which codes for transketolase,
- 12.7 the gnd gene which codes for 6-phosphogluconate
dehydrogenase,

- 12.8 the zwf gene which codes for glucose 6-phosphate dehydrogenase,
- 12.9 the lysE gene which codes for lysine export,
- 12.10 the zwal gene,
- 5 12.11 the eno gene which codes for enolase,
- 12.12 the opcA gene
- is or are amplified or over-expressed at the same time.
13. A process for the fermentative preparation of L-threonine as claimed in claim 8,
10 wherein
in coryneform microorganisms which in particular already produce L-threonine, one or more genes chosen from the group consisting of
- 15 13.1 at the same time the hom gene which codes for homoserine dehydrogenase or the hom^{dr} allele which codes for a "feed back resistant" homoserine dehydrogenase,
- 13.2 the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase,
20
- 13.3 the pyc gene which codes for pyruvate carboxylase,
- 13.4 the mqo gene which codes for malate:quinone oxidoreductase,
- 25 13.5 the tkt gene which codes for transketolase,
- 13.6 the gnd gene which codes for 6-phosphogluconate dehydrogenase,

- 13.7 the zwf gene which codes for glucose 6-phosphate dehydrogenase,
- 13.8 the thrE gene which codes for threonine export,
- 13.9 the zwal gene,
- 5 13.10 the eno gene which codes for enolase,
- 13.11 the opcA gene
- is or are amplified, in particular over-expressed, at the same time.
14. A process as claimed in claim 10,
10 wherein
for the preparation of L-amino acids, in particular L-lysine, L-threonine, L-isoleucine or L-tryptophan, bacteria in which one or more genes chosen from the group consisting of,
- 15 14.1 the pck gene which codes for phosphoenol pyruvate carboxykinase
- 14.2 the pgi gene which codes for glucose 6-phosphate6 isomerase
- 14.3 the poxB gene which codes for pyruvate oxidase or
- 20 14.4 the zwa2 gene
- is or are attenuated at the same time, are fermented.
15. A use of polynucleotide sequences as claimed in claim 1 as hybridization probes for isolation of the cDNA which codes for the tal gene product.
- 25 16. A use of polynucleotide sequences as claimed in claim 1 as hybridization probes for isolation of the cDNA or genes which have a high similarity with the sequence of the tal gene.

Figure 1:



SEQUENCE PROTOCOL

<110> National University of Ireland, Galway
Degussa-Hüls AG

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10 <140>
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/06304

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/54 C12N15/62 C12P13/08 C12Q1/68 C12P13/06
C12P13/22 //(C12P13/08,C12R1:15)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EMBL, EPO-Internal, WPI Data, CHEM ABS Data, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	UWE KÖHLER ET AL.: "Transaldolase genes from the cyanobacteria <i>Anabaena variabilis</i> and <i>Synechocystis</i> sp. PCC 6803: comparison with other eubacterial and eukaryotic homologues" PLANT MOLECULAR BIOLOGY, vol. 30, 1996, pages 213-218, XP000960916 abstract; figure 1	1,3,6, 15,16
X	JP 09 224661 A (MITSUBISHI CHEM CORP) 2 September 1997 (1997-09-02) sequence listing	1,3,6, 15,16

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

22 November 2000

Date of mailing of the international search report

01/12/2000

Name and mailing address of the ISA

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Authorized officer

Montero Lopez, B

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. Application No

PCT/EP 00/06304

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 9224661 A	02-09-1997	NONE	

Form PCT/ISA/210 (patent family annex) (July 1992)